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ZEITSCHRIFT FÜR NATURFORSCHUNG, vol. 27b, 1972, Tübingen S. MARCIANI, M. TERBOJEVIC, F. DALL'ACQUA "Light Scattering and Flow Dichroism Studies on DNA After the Photoreaction with Psoralen" pages 196-200

(73) Proprietor: Molecular Diagnostics, Inc.
400 Morgan Lane
West Haven, CT.06516 (US)

(72) Inventor: Dattagupta, Nanibhushan
470 Prospect Street
New Haven Ct 06511 (US)
Inventor: Crothers, Donald M.
Surrey Drive
Northford Ct 06472 (US)

(74) Representative: Adrian, Albert, Dr. et al
c/o BAYER AG Konzernverwaltung RP
Patentabteilung
D-5090 Leverkusen 1, Bayerwerk (DE)

(56) References cited:

MOLECULAR & GENERAL GENETICS, vol. 179, no. 1, 1980 G. VENEMA, U. CANOSI "The Effect of Trimethylpsoralen-Crosslinking on Entry of Donor DNA in Transformation and Transfection of Bacillus subtilis" pages 1-11

PHOTOCHEM PHOTOBIOL 37 363 (1983)

PROC NATL ACAD SCI US 78 6633-6637 (1981)

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Description

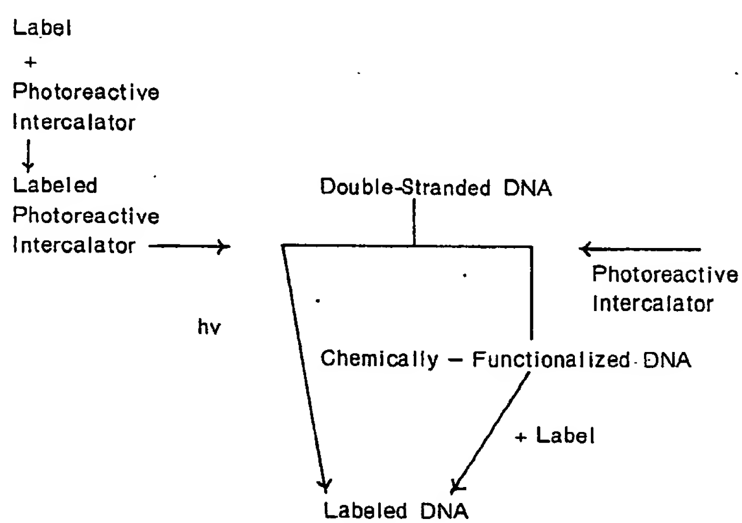
The present invention relates to a photochemical method of labelling nucleic acids for detection purposes in hybridization assays for the determination of specific polynucleotide sequences.

The most efficient and sensitive method of detection of nucleic acids such as DNA after hybridization requires radioactively labelled DNA. The use of autoradiography and enzymes makes the assay time consuming and requires experienced technical people. Recently, a non-radioactive method of labelling DNA has been described by Ward et al, European Pat. Appl. 63,879; they use the method of nick translation to introduce biotinylated U reisdue to DNA replacing T. The biotin residue is then assayed with antibiotin antibody or an avidin containing system. The detection in this case is quicker than autoradiography but the method of nick translation is a highly skilled art. Moreover, biotinylation using biotinylated UTP works only for thymine-containing polynucleotides. Use of other nucleotide triphosphates is very difficult because the chemical derivatization of A or G or C (containing —NH_2) with biotin requires elaborate and highly skilled organic chemists.

It is accordingly an object of the present invention to provide a simplified system for detection of nucleic acids by hybridization assays, which system can be easily produced and used without the disadvantages noted hereinabove.

These and other objects and advantages are realized in accordance with the present invention pursuant to which the nucleic acid is labeled by means of photochemistry, employing a photoreactive nucleic acid-binding ligand, e.g., an intercalator compound such as a furocoumarin or a phenanthridine compound or a non-intercalator compound such as netropsin, distamycin, Hoechst 33258 (chemical name: (2-(2-(4-Hydroxyphenyl)-6-benzimidazolyl)-6-(1-methyl-4-piperazyl)-benzimidazole, trihydrochloride, pentahydrate)) and bis-benzimidazole to link the nucleic acid to a label which can be "read" or assayed in conventional manner, including fluorescence detection. The end product is thus a labeled nucleic acid comprising a nucleic acid component and a label chemically linked thereto through a residue of a photochemically reactive nucleic acid binding ligand.

The novel photochemical method provides more favorable reaction conditions than the usual chemical coupling method for biochemically sensitive substances. By using proper wavelengths for irradiation, DNA, RNA and proteins can be modified without affecting the native structure of the polymers. The nucleic acid-binding ligand, hereinafter exemplified by an intercalator, and label can first be coupled and then photoreacted with the nucleic acid or the nucleic acid can first be photoreacted with the intercalator and then coupled to the label. A general scheme for coupling a nucleic acid, exemplified by a double-stranded DNA, to a label such as a hapten or enzyme is as follows:



Where the hybridizable portion of the probe is in a double stranded form, such portion is then denatured to yield a hybridizable single stranded portion. Alternatively, where the labeled DNA comprises the hybridizable portion already in single stranded form, such denaturization can be avoided if desired. Alternatively, double stranded DNA can be labeled by the approach of the present invention after hybridization has occurred using a hybridization format which generates double stranded DNA only in the presence of the sequence to be detected.

To produce specific and efficient photochemical products, it is desirable that the nucleic acid component and the photoreactive intercalator compound be allowed to react in the dark in a specific manner.

For coupling to DNA, aminomethyl psoralen, aminomethyl angelicin and amino alkyl ethidium or methidium azides are particularly useful compounds. They bind to double-stranded DNA and only the complex produces photoadduct. In the case where labeled double-stranded DNA must be denatured in order to yield a hybridizable single stranded region, conditions are employed so that simultaneous interaction of two strands of DNA with a single photoadduct is prevented. It is necessary that the frequency of modification along a hybridizable single stranded portion of the probe not be so great as to substantially prevent hybridization, and accordingly there preferably will be not more than one site of modification per 25, more usually 50, and preferably 100, nucleotide bases. Angelicin derivatives are superior to psoralen compounds for monoadduct formation. If a single-stranded probe is covalently attached to some extra double-stranded DNA, use of phenanthridium and psoralen compounds is desirable since these compounds interact specifically to double-stranded DNA in the dark. The chemistry for the synthesis of the coupled reagents to modify nucleic acids for labelling, described more fully hereinbelow, is similar for all cases.

The nucleic acid component can be singly or doubly stranded DNA or RNA or fragments thereof such as are produced by restriction enzymes or even relatively short oligomers.

The nucleic acid-binding ligands of the present invention used to link the nucleic acid component to the label can be any suitable photoreactive form of known nucleic acid-binding ligands. Particularly preferred nucleic acid-binding ligands are intercalator compounds such as the furocoumarins, e.g., angelicin (isopsoralen) or psoralen or derivatives thereof which photochemically will react with nucleic acids, e.g., 4'-aminomethyl-4,5'-dmethyl angelicin, 4'-aminomethyltrioxsalen (4'-aminomethyl-4,5',8-trimethyl-psoralen, 3-carboxy-5- or -8-amino- or -hydroxy-psoralen, as well as mono- or bis-azido aminoalkyl methidium or ethidium compounds. Photoreactive forms of a variety of other intercalating agents can also be used as exemplified in the following table:

Intercalator Classes and Representative Compounds	Literature References
Acridine dyes	
Lerman, J. Mol. Biol. 3:18(1961);	
proflavin, acridine orange, quinacrine, acriflavine	Bloomfield et al, "Physical Chemistry of Nucleic Acids", Chapter 7, pp. 429—476, Harper and Row, NY(1974) Miller et al, Biopolymers 19:2091(1980)
B. Phenanthridines	Bloomfield et al, supra
	Miller et al, supra
ethidium	
coralyne	Wilson et al, J. Med. Chem. 19:1261(1976)
ellipticine, ellipticine cation and derivatives	Festy et al, FEBS Letters 17:321(1971);
	Kohn et al, Cancer Res. 35:71(1976); LePecq et al, PNAS (USA) 71:5078(1974); Pelaprat et al, J. Med. Chem. 23:1330(1980)
C. Phenazines	Bloomfield et al, supra
5-methylphenazine cation	
D. Phenothiazines	ibid
chlopromazine	
E. Quinolines	ibid
chloroquine	
quinine	
F. Aflatoxin	ibid

Intercalator Classes and Representative Compounds	Literature References
G. Polycyclic hydrocarbons and their oxirane derivatives	ibid
3,4-benzpyrene benzopyrene diol epoxide, 1-pyrenyloxirane	Yang et al, Biochem. Biophys. Res. Comm. 82:929(1978)
benzanthracene-5,6-oxide	Amea et al, Science 176:47(1972)
H. Actinomycins actinomycin D	Bloomfield et al, supra
I. Anthracyclinones β-rhodomycin A daunamycin	ibid
J. Thixanthrenones miracil D	ibid
K. Anthramycin	ibid
L. Mitomycin	Ogawa et al, Nucl. Acids Res., Spec. Publ. 3:79(1977); Akhtar et al, Can. J. Chem. 53:2891(2975)
M. Platinum Complexes	Lippard, Accts. Chem. Res. 11:211(1978)
N. Polyintercalators echinomycin	Waring et al, Nature 252:653(1974);
	Wakelin, Biochem. J. 157:721(1976)
quinomycin triostin BBM928A tandem	Lee et al, Biochem. J. 173:115(1978): Huang et al, Biochem. 19: 5537(1980): Viswamitra et al, Nature 289:817(1981)
diacridines	LePecq et al, PNAS (USA) 72:2915(1975): Carrellakis et al, Biochim. Biophys. Acta 418:277(1976); Wakelin et al, Biochem 17:5057(1978); Wakelin et al, FEBS Lett. 104:261(1979); Capelle et al, Biochem. 18:3354(1979); Wright et al, Biochem. 19:5825(1980); Bernier et al, Biochem. J. 199:479(1981); King et al, Biochem. 21:4982(1982)
ethidium dimer	Gaugain et al, Biochem. 17:5078(1978); Kuhlman et al, Nucl. Acids Res. 5:2629(1978); Marlcovits et al, Anal. Biochem. 94:259(1979): Dervan et al, JACS 100:1968(1978); ibid 101:3664(1979).
ellipticene dimers and analogs	Debarre et al, Compt. Rend. Ser. D. 284:81(1977); Pelaprat et al, J. Med. Chem. 23:1336(1980)
heterodimers	Cain et al, J. Med. Chem. 21:658(1978); Gaugain et al, Biochem. 17:5078(1978)
trimers	Hansen et al, JCS Chem. Comm. 162(1983); Atnell et al, JACS 105:2913(1983)

Intercalator Classes and Representative Compounds	Literature References
O. Norphillin A	Loun et al, JACS 104:3213(1982)
P. Fluorenes and fluorenones	Bloomfield et al, supra
fluorenodiamines	Witkowski et al, Wiss. Beitr.-Martin-Luther-Univ. Halle Wittenberg, 11(1981)
Q. Furocoumarins	
angelicin	Venema et al, MGG, Mol. Gen. Genet. 179:1 (1980)
4,5'-dimethylangelicin	Vedaldi et al, Chem.- Biol. Interact. 36:275(1981)
psoralen	Marciani et al, Z. Naturforsch B 27(2):196(1972)
8-methoxypsoralen	Belognzov et al, Mutat. Res. 84:11(1981); Scott et al, Photochem. Photobiol. 34:63(1981)
5-aminomethyl-8-methoxy-psoralen	Hansen et al, Tet. Lett. 22:1847(1981)
4,5,8-trimethylpsoralen	Ben-Hur et al, Biochem., Biophys. Acta 331:181(1973)
4'-aminomethyl-4,5,8-tri-methylpsoralen	Issacs et al, Biochem. 16:1058(1977)
xanthotoxin	Hradecma et al, Acta Virol. (Engl. Ed.) 26:305(1982)
khellin	Beaumont et al, Biochim, Biophys. Acta 608:1829(1980)
R. Benzodipyrones	Murx et al, J. Het. Chem. 12:417(1975); Horter et al, Photochem. Photobiol. 20:407(1974)
S. Monostral Fast Blue	Juarranz et al, Acta Histochem. 70:130(1982)

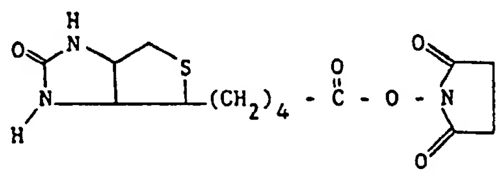
Particularly useful photoreactive forms of such intercalating agents are the azidointercalators. Their reactive nitrines are readily generated at long wavelength ultraviolet or visible light and the nitrenes of arylazides prefer insertion reactions over their rearrangement products [see White et al, Methods in Enzymol. 46:644(1977)]. Representative azidointercalators are 3-azidoacridine, 9-azidoacridine, ethidium monoazide, ethidium diazide, ethidium dimer azide [Mitchell et al, JACS 104:4265(1982)], 4-azido-7-chloroquinoline, and 2-azidofluorene. Other useful photoreactable intercalators are the furocoumarins which form [2+2] cycloadducts with pyrimidine residues. Alkylating agents can also be used such as bis-chloroethylamines and epoxides or aziridines, e.g., aflatoxins, polycyclic hydrocarbon epoxides, mitomycin, and norphillin A.

The label which is linked to the nucleic acid component according to the present invention can be any chemical group or residue having a detectable physical or chemical property. The label will bear a functional chemical group to enable it to be chemically linked to the intercalator compound. Such labeling materials have been well developed in the field of immunoassays and in general most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (see Clin. Chem.(1976)22:1243), enzyme substrates (see British Pat. Spec. 1,548,741), coenzymes (see U.S. Pat. Nos. 4,230,797 and 4,238,565), and enzyme inhibitors (see U.S. Pat. No. 4,134,792;

fluorescers (see Clin. Chem.(1979)25:353) and chromophores including phycobiliproteins; luminescers such as chemiluminescers and bioluminescers (see Clin. Chem.(1979)25:512, and ibid, 1531); specifically bindable ligands; and residues comprising radioisotopes such as ^{3}H, ^{35}S, ^{32}P, ^{125}I, and ^{14}C. Such labels are detected on the basis of their own physical properties (e.g., fluorescers, chromophores and radioisotopes) or their reactive or binding properties (e.g., enzymes, substrates, coenzymes and inhibitors). For example, a cofactor-labeled nucleic acid can be detected by adding the enzyme for which the label is a cofactor and a substrate for the enzyme. A hapten or ligand (e.g., biotin) labeled nucleic acid can be detected by adding an antibody or an antibody fragment to the hapten or a protein (e.g., avidin) which binds the ligand, tagged with a detectable molecule. Such detectable molecule can be some molecule with a measurable physical property (e.g., fluorescence or absorbance) or a participant in an enzyme rection (e.g., see above list). For example, one can use an enzyme which acts upon a substrate to generate a product with a measurable physical property. Examples of the latter include, but are not limited to, β-galactosidase, alkaline phosphatase, papain, and peroxidase. For in situ hybridization studies, ideally the final product is water insoluble. Other labels will be evident to one of the ordinary skill in the art.

The label will be linked to the intercalator compound by direct chemical linkage such as involving covalent bonds, or by indirect linkage such as by the incorporation of the label in a microcapsule or liposome which in turn is linked to the intercalator compound. Methods by which the label is linked to the intercalator compound are essentially known in the art and any convenient method can be used to perform the present invention.

Advantageously the intercalator compound is first combined with the label chemically and thereafter combined with the nucleic acid component. For example, since biotin carries a carboxyl group it can be combined with a furocoumarin by way of amide or ester formation without interfering with the photochemical reactivity of the furocoumarin or the biological activity of the biotin, e.g.,

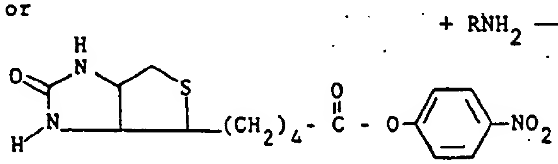


(i)

Biotin-N-hydroxysuccinimide

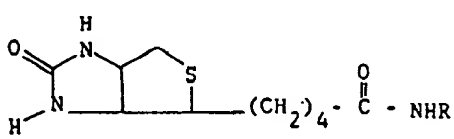
or

$$+ RNH_2 \longrightarrow$$



(ii)

Biotin-p-nitrophenyl ester

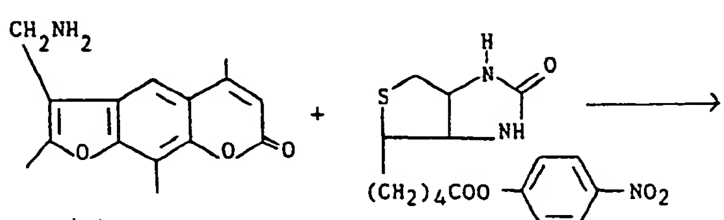


(iii)

or

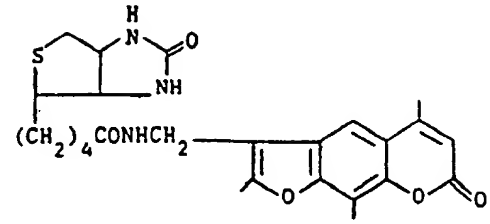
$$\text{Biotin} + \text{ROH} \xrightarrow{\text{carbodiimide}} \text{Biotin CO OR}$$

By way of example,

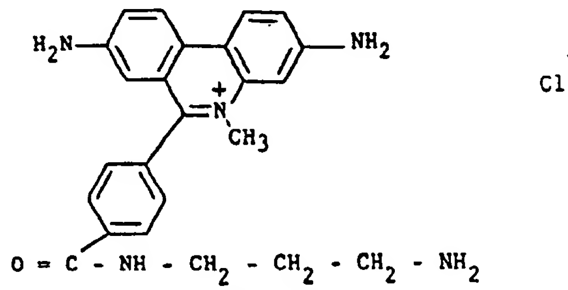


Amt

Biotin nitrophenyl ester



Other aminomethylangelicin, psoralen and phenanthridium derivatives can be similarly reacted, as can phenanthridium halides and derivatives thereof such as aminopropyl methidium chloride, i.e.



[see Hertzberg et al, J. Amer. Chem. Soc. 104:313(1982)]

Alternatively a bifunctional reagent such as dithiobis succinimidyl propionate or 1,4-butanediol diglycidyl ether can be used directly to couple the photochemically reactive molecule with the label where the reactants have alkyl amino residues, again in a known manner with regard to solvents, proportions and reaction conditions. Certain bifunctional reagents, possibly glutaraldyde may not be suitable because, while they couple, they may modify the nucleic acid and thus interfere with the assay. Routine precautions can be taken to prevent such difficulties.

The particular sequence in making the labeled nucleic acid can be varied. Thus, for example, an amino-substituted psoralen can first be photometrically coupled with a nucleic acid, the product having pendant amino groups by which it can be coupled to the label. Alternatively, the psoralen can first be coupled to a label such as an enzyme and then to the nucleic acid.

The spacer chain length between the nucleic acid-binding ligand and the label can be extended via hydrocarbon or peptide. A typical example involves extending an 8-hydroxy psoralen derivative with an alkyl halide, according to the method described by J. L. DeCout and J. Lhomme, Photochemistry Photobiology, *37*, 155—161 (1983). The haloalkylated derivative is then reacted either with thiol or amines to produce the reactive residue, as has been described by W. A. Saffran et al., Proc. Natl. Acad. Sci., U.S.A., *79*, 4594 (1982).

If the label is an enzyme, for example, the product will ultimately be placed on a suitable medium and the extent of catalysis will be determined. Thus, if the enzyme is a phosphatase the medium could contain nitrophenyl phosphate and one would monitor the amount of nitrophenol generated by observing the color. If the enzyme is a β-galactosidase the medium can contain o-nitrophenyl-D-galacto-pyranoside which also will liberate nitrophenol.

The labeled nucleic acid of the present invention is applicable to all conventional hybridization assay formats, and in general to any format that is possible based on formation of a hybridization product or aggregate comprising the labeled nucleic acid. In particular, the unique labeled probe of the present invention can be used in solution and solid-phase hybridization formats, including, in the latter case, formats involving immobilization of either sample or probe nucleic acids and sandwich formats.

The labeled nucleic acid probe will comprise at least one single stranded base sequence substantially complementary to or homologous with the sequence to be detected. However, such base sequence need not be a single continuous polynucleotide segment, but can be comprised of two or more individual segments interrupted by nonhomologous sequences. These nonhomologous sequences can be linear or they can be self-complementary and form hairpin loops. In addition, the homologous region of the probe can be flanked at the 3'- and 5'-terminii by nonhomologous sequences, such as those comprising the DNA or RNA of a vector into which the homologous sequence had been inserted for propagation. In either instance, the probe as presented as an analytical reagent will exhibit detectable hybridization at one or more points with sample nucleic acids of interest. Linear or circular single stranded polynucleotides can be used as the probe element, with major or minor portions being duplexed with a complementary polynucleotide strand or strands, provided that the critical homologous segment or segments are in single stranded form and available for hybridization with sample DNA or RNA. Useful probes include linear or circular probes wherein the homologous probe sequence is in essentially only single stranded form [see particularly, Hu and Messing, Gene 17:271(1982)].

The labeled probe of the present invention can be used in any conventional hybridization technique. As improvements are made and as conceptually new formats are developed, such can be readily applied to the present labeled probe. Conventional hybridization formats which are particularly useful include those wherein the sample nucleic acids or the polynucleotide probe is immobilized on a solid support (solid-phase hybridization) and those wherein the polynucleotide species are all in solution (solution hybridization).

In solid-phase hybridization formats, one of the polynucleotide species participating in hybridization is fixed in an appropriate manner in its single stranded form to a solid support. Useful solid supports are well known in the art and include those which bind nucleic acid either covalently or non-covalently. Noncovalent supports which are generally understood to involve hydrophobic bonding include naturally occurring and synthetic polymeric materials, such as nitrocellulose, derivatized nylon, and fluorinated polyhydrocarbons, in a variety of forms such as filters or solid sheets. Covalent binding supports are also useful and comprise materials having chemically reactive groups or groups, such as dichlorotriazine, diazobenzyloxymethyl, and the like, which can be activated for binding to polynucleotides.

A typical solid-phase hybridization technique begins with immobilization of sample nucleic acids onto the support in single stranded form. This initial step essentially prevents reannealing of complementary strands from the sample and can be used as a means for concentrating sample material on the support for enhanced detectability. The polynucleotide probe is then contacted with the support and hybridization detected by measurement of the label as described herein. The solid support provides a convenient means for separating labeled probe which has hybridized to the sequence to be detected from that which has not hybridized.

Another method of interest is the sandwich hybridization technique wherein one of two mutually exclusive fragments of the homologous sequence of the probe is immobilized and the other is labelled. The presence of the polynucleotide sequence of interest results in dual hybridization to the immobilized and labeled probe segments. See Methods in Enzymology 65:468(1980) and Gene 21:77—85(1983) for further details.

The invention will be further described in the following examples wherein parts are by weight unless otherwise expressed.

Example 1

50 mg of N-hydroxysuccinimido biotin is dissolved in 2 ml dimethylsulfoxide (soln A). 10 mg of 4′ aminomethyl trioxsalen (structure 1) (or other aminoalkyl compounds) is dissolved in 10 ml (soln B) aqueous buffer (e.g., 10 mM sodium tetraborate, pH adjusted with HCl); solution pH~8. Solution (A) and (B) are mixed in a volume ratio of 1:10 and weight ratio of 10:1, so that the activated hapten is present in large excess. The reaction is allowed to proceed at 35°C for 1 hour. The extent of the reaction is monitored by thin layer chromatography — on silica gel plates with a fluorescence indicators in a solvent 1/1/8 — methanol/acetic acid/chloroform. Under these TLC conditions unreacted aminomethyl trioxalane moves with the solvent front whereas the product has a slower mobility. Biotin does not show any fluorescence but the adduct fluoresces because of trioxsalen. Growth of the new fluorescent spot and disappearance of the original fluorescent spot indicates the extent of product formation. Since the activated biotin is in large excess, fluorescence corresponding to the starting material vanishes on TLC after the completion of reaction. Excess active biotin is reacted with glycyl-glycine or lysine. The presence of amino acid biotin product does not interfere with the photochemical reaction of psoralen-biotin compounds with DNA. Hence, a purification step after the above reaction is not essential.

Example 2

100 mg of biotin nitrophenyl ester is dissolved in dry DMSO (2—5 ml) and 10 mg of 4′-aminomethyl trioxsalen is dissolved in dry DMSO (5 ml). The two solutions are mixed in a molar ratio so that biotin nitrophenyl ester is about ten times with respect to 4′-aminomethyl trioxsalen. 100 ml of triethylamine is added to the mixture and shaken well. The progress of reaction is checked by TLC and excess unreacted biotin nitrophenyl ester is reacted with lysine as in Example 1. The reaction is allowed to proceed for 1 hour at 35°C and then lysine is added to quench the reaction. After the reaction, DMSO is evaporated under vacuum and the gummy residue is taken in methanol and can be chromatographically purified on an LH 20 column, using methanol as an eluant. The last step is not essential for the photochemical interaction of psoralen adduct with DNA.

Example 3

Biotin can be coupled to aminoalkyl hydroxyalkyl compounds by carbodiimide mediated reaction. 10 mg biotin is dissolved in 1 ml dimethyl formamide. To the solution, 5 mg of 4′-hydroxymethyl trioxsalen is added followed by 10 mg dicyclohexyl carbodiimide. The reaction is allowed to proceed for 20 hours at room temperature, dicyclohexylurea precipitate is removed and the product is recovered by removing DMF under vacuum. The same reaction can be performed in pyridine.

The foregoing examples will be give similar results if the aminoalkyl trioxsalen is replaced by other aminoalkyl furocoumarins, phenanthridium halides, and the like.

Example 4

Coupling of an enzyme to a photoactive amino compound and then covalent attachment to DNA:

A typical example is given with papain. 0.1 mg/ml of papain solution in 100 mM phosphate buffer (pH 8) is added to 10 mg/ml of amino methyl trioxsalen. The final solution should be 1:1 with respect to volume of enzyme and photoactivator solution. Then solid dithiobis-succinimidyl propionate or dimethyl suberimidate is added to a final concentration of 20 μg/ml. The pH is continuously monitored and maintained at the original value by 0.001 *M* sodium hydroxide. After adding the crosslinker twice, the reaction is allowed to proceed for 30 minutes at room temperature. The free photoactive amine is separated from the enzyme-bound compounds by gel filtration on Sephadex® G—10. The adduct is excluded along with the free protein and protein-protein conjugates. Most of these impurities have very little effect on DNA binding. Any enzyme which has been modified and still retains its activity can be coupled similarly.

After the purification, the enzyme conjugate is mixed with DNA in aqueous buffer (pH 7.5) and irradiated at 390 nm for 1 hour. The adduct is separated from the unreacted residues on Sephadex (G—100) column. The activity is tested as follows: DNA-enzyme conjugate is dialyzed against 10 mM EDTA — containing buffer (pH 6.2). To 8 ml of the DNA-enzyme solution, 10 ml of 60 mM mercaptoethanol and 1 ml 50 mmol cysteine (freshly prepared) are added. This is treated as enzyme solution. The substrate solution is prepared as follows:

592 mg benzoyl-L-arginine ethyl ester hydrochloride is dissolved in 30 ml water (BAEE).

To this BAEE solution, 1.6 ml 0.01 M EDTA, 1.6 ml 0.05 M cysteine, freshly prepared are added, pH is adjusted at 6.2 and the final volume is made up to 42 ml.

Procedure

Using a pH meter, the following test system has been set up at 25°C:

5 ml substrate
5 ml H_2O
5 ml 3 M NaCl
1 ml enzyme dilution

The amount of 0.01 M NaOH in ml required to maintain a pH of 6.2 is recorded. A five-minute period is generally satisfactory.

Since enzyme are not stable at higher temperatures, if the conjugates are used for hybridization assays, low temperatures should be used. (Either oligonucleotides, or an ionic strength less than 2 m molar should be utilized so that hybridization can be effected at low temperature.)

Example 5

Identical products are generated if aminoalkylphotoactive compounds are phororeacted with DNA first, then with the proteins or enzymes or haptens. DNA (1 mg/ml) and amino methyl trioxsalen (0.1 mg/ml) are mixed in aqueous buffer pH (7.5), and photoirradiated at 390 nm for 1 hour; the product is precipitated with ethanol then redissolved in crosslinking buffer as in Example 4, and the rest of the procedure is similar.

If monoadduct formation is essential, monoazidoaminopropyl methidium or aminomethyl angelicin compounds are used under otherwise identical conditions.

Example 6

A glycoprotein can be coupled by redox reaction to an aliphatic amine. A typical example is given below with horse radish peroxidase (HRPO) coupling to 4′ aminomethyl trioxsalen. Identical conditions can be followed with any aminoalkyl compound.

Scheme:

$$\text{HRPO} \xrightarrow{\text{Periodate}} \text{(HRPO)}-\text{CHO}$$

$$\text{(HRPO)}-\text{CHO} \xrightarrow{\text{RNH}_2} \text{(HRPO)}-\text{CH}=\text{N}-\text{R}$$

$$\text{(HRPO)}\,\text{CH}_2-\text{NHR} \xleftarrow[\text{NaBH}_4]{\text{Reduction}} \text{(HRPO)}-\text{CH}=\text{N}-\text{R}$$

Experiment:

10 mg HRPO (Sigma Chemical Co.) is dissolved in 2 ml freshly prepared 0.3 M sodium bicarbonate (pH 8.1). To the enzyme solution, 200 microliter 1% 2,4-dinitrofluorobenzene in ethanol is added to block α- and ε-amino groups and some hydroxy groups of the enzyme. The mixture is gently shaken for one hour at

room temperature. Then 2 ml 80 m molar sodium periodate in distilled water is added and mixed for 30 minutes at room temperature. In order to quench the unreacted periodate, ethylene glycol is added to a final concentration of 50 m Mol. The solution is dialyzed againt 10 m molar sodium carbonate buffer (pH 9.5) in a cold room (~4°C). To the dialyzed solution, ~1 mg solid aminomethyl trioxsalen is added and the mixture is shaken gently for 1 hour at 25°C. 10 mg sodium borohydride ($NaBH_4$) solid is added and the reaction is allowed to proceed for 12 hours at 4°C. The adduct is dialyzed against the DNA binding buffer and then photoreacted by mixing in 1:1 weight ratio (enzyme to DNA) as described before. The separation of the DNA-enzyme adduct from the enzyme is done by gel filtration on a Sephadex G—100 column where the adduct is excluded.

To improve the photochemical efficiency, blocking of reactive HRPO sites before oxidation with periodate may be done with allylisothiocyanate, as has been described by P. K. Nakane et al, Enzyme Labeled Antibodies for Light and Electron Microscopic Localization of Antigens, J. Histochem Cytochem, *14*. 790 (1966).

Unless stated otherwise, all the reactions are performed in the dark or red light conditions are maintained.

The peroxidase activity is measured by the following method:

100—500 microliters of the sample are mixed with 3 ml 14 mM para-Cresol in 50 m molar tris HCl buffer (pH 7.5). To this 1 ml 1% H_2O_2 is added. After 2 minutes, 3 ml 5 m molar sodium cyanides in water are added to quench the reaction. The fluorescence of the solution is measured at excitation 320 nm, emission 410 nm. H. Perschke and E. Broda, Nature *190*, 257 (1961); M. Roth, Methods of Biochemical Analysis, vol. 17, ed. D. Glick, Interscience Publisher, N.Y., 1969, P. 236.

Example 7

Assay for the label after DNA—DNA hybridization:

An illustrative example with a single stage DNA—DNA hybridization is presented here. The procedure used in the case of two-stage hybridization as described in European Patent Application No. 84 107 248.1 published as EP—A—0 130 515 can also be followed. Plasmid pBR322 (New England Biolab) is digested with the restriction endonuclease, Pst 1 and Pvu 1. This double digestion produces one fragment of 126 base pair long DNA containing the part of ampicillin resistance gene and another fragment of 4236 base pair long DNA. The 126 bp long fragment is isolated by running the double digest on 5% polyacrylamide gel. A part of this DNA is labeled either with biotin or with enzymes as described before and used as the labeled probe. For hybridization, Pst 1 cut pBR322 (for control) or the test sample DNA is covalently linked to cellulose by photochemical method as described in European Patent Application No. 84 107 266.3 published as EP—A—0 130 523, by cyanogen bromide activation or by diazotization method (H. Bünemann, Nucleic Acids Res., *10*, 7181 (1982)).

The cellulose containing the denatured DNA is suspended in 5 m molar salt solution for hybridization with enzyme-coupled DNA or suspended in 2.4 *M* tetraethylammonium chloride when biotinylated DNA is used. Then hybridization is done as described by H. Bünemann in Nucleic Acids Research, *10*, 7181 (1982) for the detection of the ampicillin resistance gene using 126 base pairs labeled fragment as the probe. In low salt, hybridization is done at 30—40°C; in 2.4 *M* (high salt), it is done between 40 and 50°C.

After hybridization, FITC-labelled avidine is used to assay for biotin or proper enzyme assay is done with the particles.

Claims for the Contracting States: BE CH DE FR GB IT LI LU NL SE

1. A labeled nucleic acid comprising a nucleic acid component and a label chemically linked thereto, characterized in that the label is chemically linked to the nucleic acid component through a residue of a photochemically reactive nucleic acid binding ligand.

2. The labeled nucleic acid of Claim 1, characterized in that the nucleic acid binding ligand is an intercalator compound.

3. The labeled nucleic acid of Claim 2, characterized in that the intercalator compound is selected from acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines, and quinolines.

4. The labeled nucleic acid of any of Claims 1 to 3, characterized in that the label is a specifically bindable ligand, preferably biotin or a hapten, an enzyme, a phycobiliprotein, or a fluorescent or luminescent radical, preferably a radical of fluorescin.

5. The labeled nucleic acid of any of Claims 1 to 4, characterized in that the nucleic acid component is substantially entirely in single stranded form.

6. The labeled nucleic acid of any of Claims 1 to 5, characterized in that the nucleic acid component comprises a double stranded portion.

7. An adduct suitable for photochemical attachment to a nucleic acid probe comprising a nucleic acid reactive component and a label chemically linked thereto, characterized in that the nucleic acid reactive component is a photochemically reactive nucleic acid binding ligand.

8. The adduct of Claim 7, characterized in that the nucleic acid binding ligand is an intercalator compound, preferably selected from acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines, and quinolones.

9. A method for preparing a labeled nucleic acid, characterized in that the nucleic acid to be labeled is contacted with the adduct of Claim 7 or 9 and the mixture subjected to photochemical irradiation.

10. A method for preparing a labeled nucleic acid, characterized in that the nucleic acid to be labeled is contacted with a photochemically reactive nucleic acid binding ligand, the mixture is subjected to photochemical irradiation to covalently link the nucleic acid and the binding ligand, and thereafter the reaction product is reacted with a label to chemically link such label to the reaction product.

11. The method of Claim 10, characterized in that the nucleic acid binding ligand is an intercalator compound, preferably selected from acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolones.

12. The use of the labeled nucleic acid of any of Claims 1 to 6 in a nucleic acid hybridization assay.

Claims for the Contracting State: AT

1. A method for preparing a labeled nucleic acid, characterized in that the nucleic acid to be labeled is contacted with a photochemically reactive nucleic acid binding ligand, the mixture is subjected to photochemical irradiation to covalently link the nucleic acid and the binding ligand, and thereafter the reaction product is reacted with a label to chemically link such label to the reaction product.

2. The method of Claim 1, characterized in that the nucleic acid binding ligand is an intercalator compound.

3. The method of Claim 2, characterized in that the intercalator compound is selected from acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolones.

4. The method of any of Claims 1 to 3, characterized in that the label is a specifically bindable ligand, preferably biotin or a hapten, an enzyme, a phycobiliprotein, or a fluorescent or luminescent radical, preferably a radical of fluorescin.

5. The method of any of Claims 1 to 4, characterized in that the nucleic acid component is substantially entirely in single stranded form.

6. The method of any of Claims 1 to 5, characterized in that the nucleic acid component comprises a double stranded portion.

7. Use of a labeled nucleic acid comprising a nucleic acid component and a label chemically linked thereto in a nucleic acid hybridization assay, characterized in that the label is chemically linked to the nucleic acid component through a residue of a photochemical reactive nucleic acid binding ligand.

Patentansprüche für die Vertragsstaaten: BE CH DE FR GB IT LI LU NL SE

1. Markierte Nukleinsäure aus einer Nukleinsäurekomponente und einer chemisch daran gebundenen Markierung, dadurch gekennzeichnet, daß die Markierung an die Nukleinsäurekomponente durch einen Rest eines photochemisch reaktiven Nukleinsäurebindungsliganden an die Nukleinsäure gebunden ist.

2. Markierte Nukleinsäure gemäß Anspruch 1, dadurch gekennzeichnet, daß der Nukleinsäurebindungsligand eine Einschlußverbindung ist.

3. Markierte Nukleinsäure gemäß Anspruch 2, dadurch gekennzeichnet, daß die Einschlußverbindung ausgewählt ist aus Acridinfarbstoffen, Phenanthridinen, Phenazinen, Furokumarinen, Phenothiazinen und Chinolonen.

4. Markierte Nukleinsäure gemäß einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß die Markierung ein zu einer spezifischen Bindung befähigter Ligand, vorzugsweise Biotin oder ein Hapten, ein Enzym, ein Phycobiliprotein oder ein fluoreszierender oder lumineszierender Rest, vorzugsweise ein Fluoreszeinrest ist.

5. Markierte Nukleinsäure gemäß einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß die Nukleinsäurekomponente im wesentlichen vollständig in Einzelstrangform vorliegt.

6. Markierte Nukleinsäure gemäß einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß die Nukleinsäurekomponente einen Doppelstranganteil umfaßt.

7. Ein für eine photochemische Anhaftung an eine Nukleinsäureverbindung geeignetes Addukt aus einer Nukleinsäurereaktiven Verbindung und einer chemisch daran gebundenen Markierung, dadurch gekennzeichnet, daß die Nukleinsäurereaktive Komponente ein photochemisch reaktiver Nukleinsäurebindungsligand ist.

8. Addukt gemäß Anspruch 7, dadurch gekennzeichnet, daß der Nukleinsäurebindungsligand eine Einschlußverbindung ist, die vorzugsweise aus Acridinfarbstoffen, Phenanthridinen, Phenazinen, Furokumarinen, Phenothiazinen und Chinolonen ausgewählt ist.

9. Verfahren zur Herstellung einer markierten Nukleinsäure, dadurch gekennzeichnet, daß die zu markierende Nukleinsäure mit dem Addukt gemäß Ansprüchen 7 oder 8 in Berührung gebracht wird und man die Mischung einer photochemischen Bestrahlung aussetzt.

10. Verfahren zur Herstellung einer markierten Nukleinsäure, dadurch gekennzeichnet, daß die zu markierende Nukleinsäure mit einem photochemisch-reaktiven Nukleinsäure-bindenden Liganden in Berührung gebracht wird, daß man die Mischung einer photochemischen Bestrahlung unterwirft, um die Nukleinsäure und den Bindungsliganden kovalent zu verbinden und man anschließend das Reaktionsprodukt mit einer Markierung umsetzt, um diese Markierung an das Reaktionsprodukt chemisch zu binden.

11. Verfahren gemäß Anspruch 10, dadurch gekennzeichnet, daß der Nukleinsäurebindungsligand eine Einschlußverbindung ist, die vorzugsweise aus Acridinfarbstoffen, Phenanthridinen, Phenazinen, Furokumarinen, Phenothiazinen und Chinolonen ausgewählt ist.

12. Verwendung der markierten Nukleinsäure gemäß einem der Ansprüche 1 bis 6 in einem Nukleinsäurehydridisierungsassay.

Patentansprüche für den Vertragsstaat: AT

1. Verfahren zur Herstellung einer markierten Nukleinsäure, dadurch gekennzeichnet, daß die zu markierende Nukleinsäure mit einem photochemisch-reaktiven Nukleinsäure-bindenden Liganden in Berührung gebracht wird, daß man die Mischung einer photochemischen Bestrahlung unterwirft, um die Nukleinsäure und den Bindungsliganden kovalent zu verbinden und man anschließend das Reaktionsprodukt mit einer Markierung umsetzt, um diese Markierung an das Reaktionsprodukt chemisch zu binden.

2. Verfahren gemäß Anspruch 1, dadurch gekennzeichnet, daß der Nukleinsäure-bindende Ligand eine Einschlußverbindung ist.

3. Verfahren gemäß Anspruch 2, dadurch gekennzeichnet, daß die Einschlußverbindung ausgewählt ist aus Akridinfarbstoffen, Phenanthridinen, Phenazinen, Furokumarinen, Phenothiazinen und Chinolonen.

4. Verfahren gemäß einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß die Markierung ein zu einer spezifischen Bindung befähigter Ligand, vorzugsweise Biotin oder ein Hapten, ein Enzym, ein Phycobiloprotein oder ein fluoreszierender oder lumineszierender Rest, vorzugsweise Fluoreszeinrest ist.

5. Verfahren gemäß einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß die Nukleinsäurekomponente im wesentlichen vollständig in Einzelstrangform vorliegt.

6. Verfahren gemäß einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß die Nukleinsäurekomponente einen Doppelstranganteil umfaßt.

7. Verwendung einer markierten Nukleinsäure aus einer Nukleinsäurekomponente und einer daran chemisch gebundenen Markierung in einem Nukleinsäurehybridisierungsassay, dadurch gekennzeichnet, daß die Markierung chemisch an die Nukleinsäurekomponente mittels eines Restes aus einem photochemisch-reaktiven Nukleinsäurebindungsliganden gebunden ist.

Revendications pour les Etats contractants: BE CH DE FR GB IT LI LU NL SE

1. Acide nucléique marqué, comprenant un composant acide nucléique et un marqueur chimiquement lié à ce composant, caractérisé en ce que le marqueur est lié chimiquement au composant acide nucléique par un résidu de ligand de liaison à l'acide nucléique photochimiquement réactif.

2. Acide nucléique marqué suivant la revendication 1, caractérisé en ce que le ligand de liaison à acide nucléique est un composé d'intercalation.

3. Acide nucléique marqué suivant la revendication 2, caractérisé en ce que le composé d'intercalation est choisi entre des colorants d'acridine, des phénanthridines, des phénazines, des furocoumarines, des phénothiazines et des quinoléines.

4. Acide nucléique marqué suivant l'une quelconque des revendications 1 à 3, caractérisé en ce que le marqueur est un ligand susceptible de liaison spécifique, de préférence la biotine ou un naphtène, un enzyme, une phycobiliprotéine ou un radical fluorescent ou luminescent, de préférence un radical de fluorescéine.

5. Acide nucléique marqué suivant l'une quelconque des revendications 1 à 4, caractérisé en ce que le composant acide nucléique est à peu près entièrement sous la forme monocaténaire.

6. Acide nucléique marqué suivant l'une quelconque des revendications 1 à 5, caractérisé en ce que le composant acide nucléique comprend une portion bicaténaire.

7. Produit d'addition convenant pour la fixation photochimique à une sonde d'acide nucléique, comprenant un acide nucléique réactif et un indicateur chimiquement lié à lui, caractérisé en ce que le composant réactif acide nucléique est un ligand de liaison à l'acide nucléique photochimiquement réactif.

8. Produit d'addition suivant la revendication 7, caractérisé en ce que le ligand de liaison à l'acide nucléique est un composé d'intercalation, choisi de préférence entre des colorants d'acridine, des phénanthridines, des phénazines, des furocoumarines, des phénothiazines et des quinolones.

9. Produit de préparation d'un acide nucléique marqué, caractérisé en ce que l'acide nucléique qui doit être marqué est mis en contact avec le produit d'addition suivant la revendication 7 ou 8 et le mélange est soumis à une irradiation photochimique.

10. Procédé de préparation d'un acide nucléique marqué, caractérisé en ce que l'acide nucléique devant être marqué est mis en contact avec un ligand de liaison à l'acide nucléique photochimiquement réactif, le mélange est soumis à une irradiation photochimique pour attacher par covalence l'acide nucléique et le ligand de liaison, puis le produit de réaction est amené à réagir avec un marqueur pour attacher chimiquement ce marqueur au produit de réaction.

11. Procédé suivant la revendication 10, caractérisé en ce que le ligand de liaison à l'acide nucléique est un composé d'intercalation choisi de préférence entre des colorants d'acridine, des phénanthridines, des phénazines, des furocoumarines, des phénothiazines et des quinolones.

12. Utilisation de l'acide nucléique marqué suivant l'une quelconque des revendications 1 à 6, dans un essai d'hybridation d'acide nucléique.

Revendications pour l'Etat contractant: AT

1. Procédé de préparation d'un acide nucléique marqué, caractérisé en ce que l'acide nucléique devant être marqué est mis en contact avec un ligand de liaison à l'acide nucléique photochimiquement réactif, le mélange est soumis à une irradiation photochimique pour attacher par covalence l'acide nucléique et le ligand de liaison, puis le produit réactionnel est amené à réagir avec un marqueur pour attacher chimiquement ce marqueur au produit de réaction.

2. Procédé suivant la revendication 1, caractérisé en ce que le ligand de liaison à l'acide nucléique est un composé d'intercalation.

3. Procédé suivant la revendication 2, caractérisé en ce que le composé d'intercalation est choisi entre des colorants d'acridine, des phénanthridines, des phénazines, des furocoumarines, des phénothiazines et des quinolones.

4. Procédé suivant l'une quelconque des revendications 1 à 3, caractérisé en ce que le marqueur est un ligand susceptible de liaison spécifique, de préférence la biotine ou un haptène, un enzyme, une phycobiliprotéine ou un radical fluorescent ou luminescent, de préférence un radical de fluorescéine.

5. Procédé suivant l'une quelconque des revendications 1 à 4, caractérisé en ce que le composant acide nucléique est à peu près entièrement sous la forme monocaténaire.

6. Procédé suivant l'une quelconque des revendications 1 à 5, caractérisé en ce que le composant acide nucléique comprend une portion bicaténaire.

7. Utilisation d'une acide nucléique marqué comprenant un composant acide nucléique et un marqueur chimiquement lié à ce composant dans un essai d'hybridation d'acide nucléique, caractérisé en ce que le marqueur est lié chimiquement au composant acide nucléique par un résidu d'un ligand de liaison à l'acide nucléique photochimiquement réactif.